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An Improved Method for the Determination of Aryl-4-hydroxylase Activity

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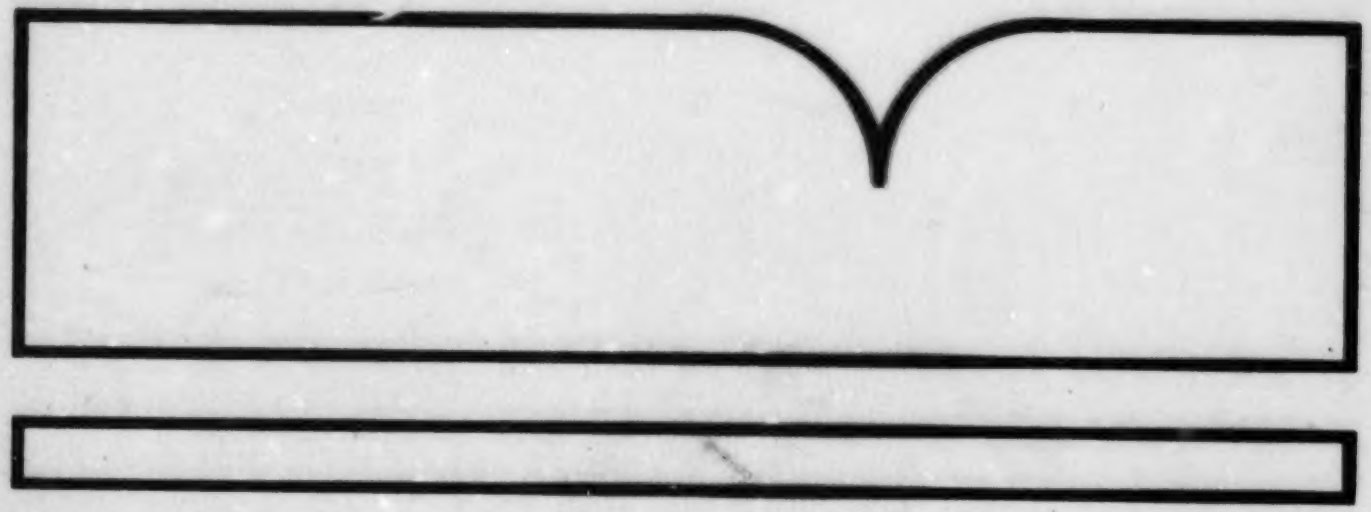

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An Improved Method for the Determination of Aryl-4-hydroxylase Activity

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An improved method for separation of tritiated benzo[*a*]pyrene from tritiated water in the aryl-4-hydroxylase assay is presented. Quantitative retention of benzo[*a*]pyrene was obtained with mixed cellulose acetate and nitrate filters. Enzymatic activities obtained with this modification agreed well with those determined using the standard charcoal column extraction. This modification greatly reduces the time required to perform this assay, and allows for the determination of enzyme activity in a large number of samples.

INTRODUCTION

The introduction of S-9 metabolic activation system into genetic toxicology[1] has produced increased interest in quantitating aryl-4-hydroxylase activity (EC 1.14.14.1). Aryl-4-hydroxylase activity has been determined by either a fluorescent method[2] or a tritium release assay.[3] The tritium release method is simple, rapid, and avoids the problems inherent in fluorometric determinations. However, this method involves a separation of substrate from free tritium using charcoal columns, which, although efficient, requires a substantial amount of time. Studies in this laboratory indicate that mixed cellulose acetate and nitrate filters quantitatively retain certain organic compounds filtered from aqueous solutions (E. Davey, unpublished data). This paper describes the use of mixed cellulose acetate and nitrate filters as an improved separation technique for the tritium release aryl-4-hydroxylase assay.

EXPERIMENTAL

Mixed cellulose acetate and nitrate filters, pore size 0.22 μm, were obtained from Millipore Corp., Bedford, Massachusetts,‡ with [1,3,6-^{3}H] benzo[*a*]pyrene§ (B[*a*]P), spec. act. 66.3 Ci mmol^{-1}, and Aquasol-2 purchased from New England Nuclear, Boston, Massachusetts. Non-radioactive benzo[*a*]pyrene (+99%) was obtained from Aldrich Chemical Corp., Milwaukee, Wisconsin. All hydroxylase assays were performed using F-12 (Ham's) medium (Grand Island Biological Co., Grand Island, New York) as a diluent. Rat liver S-9 (Arachlor 1254 induced) was obtained from Litton Bionetics, Bethesda, Maryland, with NADP and isocitric acid purchased from Sigma Chemical Co., St. Louis, Missouri.

Aryl-4-hydroxylase assay

The aryl-4-hydroxylase determinations were performed as previously reported[3] except as noted. Assays were performed in 15 ml disposable conical tubes (Falcon, Oxnard, California) at a final volume of 1 ml. Aliquots of rat liver S-9 and cofactor solution, 1 nmol NADP per μg S-9 protein and 6 nmol isocitric acid per μg S-9 protein, were added to each tube and diluted to 980 μl with media. F-12 (Ham's) medium was employed as a diluent in these aryl-4-hydroxylase assays in order to duplicate the conditions used in cell culture mutation assays. 20 μl of tritiated B[*a*]P (spec. act. 7.79 mCi mmol^{-1}, 5 mM) was added to each tube followed by incubation for 10 min at 37 °C. The reaction was halted by the addition of 50 μl of 4 N acetic acid, followed by placing on ice.

The reaction mix was filtered through mixed cellulose acetate and nitrate filters using a 12 sample filtering manifold (Millipore Corp., Bedford, Massachusetts). The filters were washed with 2 × 1 ml aliquots of deionized water, with the filtrate collected in test tubes. A 0.5 ml sample of filtrate was added to 10 ml of Aquasol-2 and the radioactivity determined.

Using this filtering technique, aryl-4-hydroxylase activity was determined in rat liver S-9 preparations over a range of protein concentrations between 0.25 and 1.25 mg ml^{-1}. The comparability of this method with the use of charcoal separation was further examined. The charcoal columns were prepared as described,[3] and the aryl-4-hydroxylase activity determined over a 5-fold range of rat liver S-9 protein concentration.

The effects of several B[*a*]P diluting mixtures on retention were determined by applying the B[*a*]P with deionized water, F-12 media, and acidified F-12 media (50 μl 4 N acetic acid per ml).

RESULTS

Table 1 presents the retention of B[*a*]P over a 10-fold concentration range. This range includes the range of B[*a*]P concentrations used for most aryl-4-hydroxylase determinations. The filters consistently retained more than 98% of

† Author to whom correspondence should be addressed.
‡ Mention of specific products does not constitute endorsement by the US Environmental Protection Agency.
§ Benzo[*a*]pyrene is listed on the Category I OSHA List of Carcinogens. Handle with appropriate caution.

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Table 1. Benzo[*a*]pyrene retention on mixed cellulose acetate and nitrate filters

Quantity applied (μmol)[a]	% retained
200	98.94
100	98.92
50	98.58
20	98.38

[a] Appropriate volumes of a 5 mM tritiated B[*a*]P solution in acetone were diluted to 1 ml with acidified media and filtered as described.

the applied B[*a*]P. As presented in Fig. 1, this method yielded a linear relationship between tritium release and quantity of enzyme present, with a small range between duplicates.

As shown in Fig. 2, the first method and the charcoal column method compared very well, with a correlation coefficient of $r = 0.98$. The mixed cellulose acetate and nitrate filtration method yielded consistently higher values at all protein concentrations.

It was noted that when B[*a*]P was applied with deionized water, only 81.32% of the radioactivity was retained as opposed to 97.5% with F-12 media and 98.6% with acidified F-12 media.

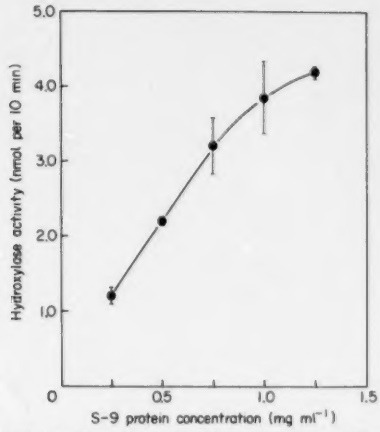

Figure 1. Aryl-4-hydroxylase activity of commercially prepared rat liver S-9. Mean ± range of duplicate analyses.

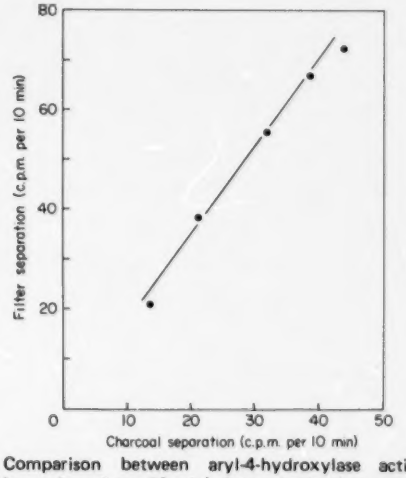

Figure 2. Comparison between aryl-4-hydroxylase activity (as c.p.m. tritium released per 10 min) determined either with charcoal column or filter procedures.

DISCUSSION

The use of mixed cellulose acetate and nitrate filters has several advantages over charcoal columns. Filtration can be performed in a few minutes using a disposable, commercially available product. The number of samples analyzed at one time can readily be increased by the addition of filtering manifolds to the vacuum system. The charcoal columns must be individually packed, which produces some variability in flow rates. In addition, the columns are of limited lifespan, necessitating frequent replacement of columns.

A primary requirement for the applicability of this membrane filtration method to the enzymatic assay is the ability of the filters to efficiently retain B[*a*]P. The results clearly indicate that the filters retain B[*a*]P efficiently and with good reproducibility.

It was noted that incomplete B[*a*]P retention by the filters occurred if the compound was diluted with deionized water rather than with media. This implies the necessity of an appropriate ionic environment for the proper retention of the compound by the filters. This is not a practical problem since aryl-4-hydroxylase assays, even without media, contain sufficient solutes to allow for efficient extraction. The results showing the filter method yielding a consistently higher value at all protein concentrations should be attributed to tritium retained in the charcoal bed volume water.

It was demonstrated that mixed cellulose acetate and nitrate filters are able to quantitatively extract B[*a*]P from aqueous solutions. This property could be applied to the use of filtration to the tritium release method for aryl-4-hydroxylase determination. This alternative procedure produces values for activity which agree well with values from the charcoal columns without the disadvantages.

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